The Influence of Treatment with Pegylated Interferon-Alfa and Ribavirin on Neutrophil Function and Death in Patients with HIV/HCV Coinfection

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Abstract

In patients with human immunodeficiency virus (HIV) as well as in patients with hepatitis C virus (HCV) infection the impairment of neutrophil activity is observed. We decided to analyze how treatment with pegylated interferon-alfa (Peg-IFN-alfa) and ribavirin affects neutrophil function in HIV/HCV coinfecting patients. The study group consisted of 18 patients with HIV/HCV coinfection, on combination antiretroviral treatment (cART), aged between 27 and 42 y (mean 33.1 ± 4.5 y). At the beginning of treatment with Peg-IFN-alfa and ribavirin all patients had an undetectable HIV viral load, and CD4 T-cell counts higher than 350 cells/ μ L. At two time points, before and after 12 wk of treatment with Peg-IFN-alfa and ribavirin, we examined intracellular levels of reactive oxygen species (ROS), and expression of selected adhesion molecules on whole blood neutrophils, along with apoptosis and necrosis of these cells. These analyses were done with flow cytometry. During anti-HCV therapy undetectable HIV levels were maintained in all patients. Treatment with PEG-IFN-alfa and ribavirin resulted in increases in the expression of CD11b and CD18, and decreases of CD16 and CD62L. However, only the change in CD62L expression was statistically significant ($p < 0.05$). Moreover, the treatment resulted in increased apoptosis of neutrophils, while necrosis remained unchanged. After 12 wk of treatment, an increase in ROS production by neutrophils stimulated with PMA was observed ($p < 0.01$). In HIV/HCV coinfecting patients on cART, PEG-IFN-alfa and ribavirin treatment caused an activation of neutrophil function, yet it did not affect the suppression of HIV replication.

Introduction

IN EUROPE AND THE U.S., ABOUT ONE-THIRD OF HUMAN IMMUNODEFICIENCY VIRUS (HIV)-POSITIVE PEOPLE are coinfected with hepatitis C virus (HCV) via the same route of transmission (24,26). It is well known that the prognosis of individuals with coinfection is worse than in HCV mono-infected patients. In coinfecting patients progression of liver fibrosis occurs more rapidly, and patients are more likely to develop liver cirrhosis and liver carcinoma (1,18). Also, the response to treatment with pegylated-interferon-alfa (Peg-IFN-alfa) and ribavirin in coinfecting patients is not as good as in individuals infected with HCV alone (16). Antiviral treatment with Peg-IFN-alfa and ribavirin eliminates HCV in less than half of coinfecting patients (22,28).

Interferon- α (IFN- α) displays antiviral, immunoregulatory, and antiproliferative activity (23,27). This cytokine stimulates transcriptional activation of hundreds of genes with antiviral

efficacy. IFN- α enhances production of reactive oxygen species (ROS), increases expression of adhesive molecules, and participates in the regulation of apoptosis (2,4,19). Being the key second messengers, ROS are involved in numerous signaling pathways, and they modulate phagocytosis, secretion, gene expression, and apoptosis (10). Augmented ROS production in patients with chronic hepatitis C can contribute to the inhibition of HCV replication and thus to the elimination of HCV (6). However, excessive ROS production can cause hepatocyte injury (7,9).

In a previous study we observed that as a result of treatment with IFN- α and thymus factor X (TFX), the formation of free oxygen radicals by resting (unprimed) neutrophils increased both without stimulation and following stimulation by N-formyl-methionyl-leucyl-phenylalanine (fMLP). Along with these changes, a compensatory increase in serum anti-oxidative capacity was observed (14). We decided to analyze the effect of Peg-IFN-alfa and ribavirin treatment on

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polymorphonuclear leukocytes (PMNs) oxygen metabolism, the expression of adhesion molecules CD11b, CD16, CD18, and CD62L, and the death of neutrophils in patients with HIV/HCV coinfection.

Materials and Methods

The study group included 18 patients with HIV/HCV coinfection, and the control group consisted of 34 patients with HCV monoinfection. Patients with other systemic or inflammatory diseases, liver cirrhosis, other causes of liver disease, previous immunosuppressive or anti-HCV treatment, and pregnant women were excluded from this study. The diagnosis of chronic hepatitis was based on the results of liver biopsy specimen examination. HCV infection was established based on the presence of viral genetic material detected by the RT-PCR method. Combined therapy using Peg-IFN-alfa 2a (Pegasys; Roche, Basel, Switzerland) and ribavirin was applied. Pegasys was administered subcutaneously once a week in a dose of 180 μ g. Ribavirin was administered orally daily in a dose dependent on the patient's weight (those less than 60 kg received 1000 mg, and those above 60 kg received 1200 mg). Upon the introduction of Peg-IFN-alfa with ribavirin, all patients had received anti-retroviral treatment and had undetectable HIV viremia (less than 50 copies/mL). Before and after 12 wk of treatment with Peg-IFN-alfa and ribavirin, intracellular ROS levels, the expression of adhesion molecules CD11b/MAC-1, CD16, CD18, and CD62L on neutrophils, as well as apoptosis and necrosis of these cells were analyzed with the use of flow cytometry. Out of 18 patients who began treatment with Peg-IFN-alfa and ribavirin, 15 continued the treatment for at least 12 wk, and were subjected to analysis of the studied parameters at two time points (before treatment and at week 12 of treatment), whereas in three patients these parameters were assessed solely before treatment.

Flow cytometry

Flow cytometric analyses were performed on heparinized whole blood (Li-Heparin, Monovette Blood Collection System; Sarsted, Nümbrecht, Germany) with the use of a FACS Canto II Flow Cytometer (Becton Dickinson, San Jose, CA). Granulocytes were gated using forward and side scatter (FSC/SSC) dot plots. Ten thousand events within the granulocyte gate were counted per sample. The data were analyzed using FACS Diva software (Becton Dickinson). Prior to each analysis, the flow cytometer was calibrated by BD Cytometer Setup & Tracking Beads (BD Biosciences, San Jose, CA). The percentage of positively labeled cells as well as mean fluorescence intensity (MFI) were used to quantify the effects.

Determination of CD11b/MAC-1, CD16, CD18, and CD62L expression

A sample of 50 μ L of heparinized blood was incubated with monoclonal mouse anti-human antibody against specific surface antigens: CD11b-PE (clone D12.11), CD62L-PE (SK11), CD16-FITC (NKP15i), and CD18-FITC (L130) (all provided by BD Pharmingen, San Jose, CA) for 30 min at room temperature in the dark. Then the erythrocytes were lysed with BD FACS Lysing Solution (BD Biosciences) for 10 min. After lysis the cells were washed once in BD Cell-

WASH solution and resuspended in BD CellFIX solution (BD Biosciences).

Assessment of ROS production

Intracellular ROS levels were measured with the use of a commercially available assay kit (Phagoburst; Orpegen Pharma, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 100 μ L of blood was transferred into 5-mL polypropylene FACS tubes and pre-incubated for 10 min in an ice cold water bath. The sample of blood was then mixed with plain buffer (negative control) or one of the following stimulants: phorbol 12-myristate 13-acetate (PMA, final concentration: 1.35 μ M) or a suspension of opsonized *Escherichia coli* ($0.17\text{--}0.33 \times 10^9$ bacteria per mL). The tubes were incubated for 10 min at 37°C in a water bath. After that, the cells were incubated with dihydrorhodamine 123 (DHR 123) for 10 min at 37°C. After lysing of erythrocytes and washing, DNA staining solution was added to exclude aggregation artifacts of bacteria or cells during the flow cytometric analysis.

Cell death analysis

Apoptosis and necrosis of granulocytes was measured using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). According to the manufacturer's protocol, blood erythrocytes were lysed by incubation of 200 μ L of blood with BD Pharm Lyse Lysing Buffer for 10 min at room temperature. After washing, the leukocytes were resuspended in Annexin V Binding Buffer at a concentration of 1×10^6 cells/mL. One hundred microliters of cell solution (1×10^5 cells/mL) was transferred into FACS tubes and incubated for 15 min at room temperature with FITC-conjugated Annexin-V and propidium iodide (PI). After adding 400 μ L of Annexin V Binding Buffer, the samples were measured on a flow cytometer within 30 min. Apoptotic cells were defined as cells demonstrating positive staining for Annexin-V-FITC [Annexin V(+); with negative or positive PI staining, respectively, for early and late stages of apoptosis], while necrotic cells were PI-positive [PI(+)] and Annexin-V-FITC-negative. Viable cells were defined as double negative for both stains.

Statistical analysis

The results are presented as arithmetic mean $\pm$ standard deviation (mean $\pm$ SD) for the normally distributed parameters, or as a median and interquartile range [median (Q1–Q3)] for data showing a departure from normality (the assumption of normality was assessed with the Shapiro-Wilk test). An independent samples *t*-test was conducted to compare the normally distributed unpaired data sets. Statistical comparisons of non-normally distributed variables were performed either with the Mann-Whitney *U* test or the Wilcoxon signed-rank test (for independent and paired data, respectively). A chi-square test was used for testing an association between categorical variables.

Results

Profile of the patients

There were no statistically significant differences in age, gender, ALT and GTP activity, HCV viral load, or

TABLE 1. CHARACTERISTICS OF THE PATIENTS

Parameter	HIV/HCV coinfection (n = 18)	HCV infection (n = 34)
Age (y)	33.1 ± 4.5	33.5 ± 12.3
Male gender	53.3%	61.8%
ALT (U/L)	88.4 ± 61.0	80.3 ± 68.8
GPT (U/L)	87.2 ± 37.8	60.3 ± 33.7
HCV viremia (IU/mL × 10 ³)	3351 ± 3098	2832 ± 2521
Histopathology staging (S) ^a	1.9 ± 0.8	2.1 ± 0.9
Histopathology grading (G) ^a	1.6 ± 0.8	1.3 ± 1.1

^aEstimation according Batt and Ludwig's scale.

GPT, glutamic-pyruvic transaminase; HCV, hepatitis C virus; ALT, alanine aminotransferase; HIV, human immunodeficiency virus.

histopathological changes between the group of HIV/HCV coinfecting patients and the control group (patients with HCV monoinfection) (Table 1). Before antiviral treatment, expression of CD11b/MAC-1, CD16, CD18, and CD62L antigens, and ROS production, as well as the percentage of apoptotic and necrotic whole blood neutrophils of HIV/HCV coinfecting patients did not differ significantly from the group of patients with HCV monoinfection (Table 2). At the 12th week of antiviral therapy, the HCV viral load in HIV/HCV coinfecting patients decreased significantly, from 2580 (549–6415) IU/mL (× 10³) to 0.002 (0.002–374) IU/mL (× 10³; *p* < 0.0001). During treatment with Peg-IFN- α and ribavirin all HIV/HCV patients were on combination antiretroviral therapy (cART) and had undetectable HIV viremia (less than 50 copies/mL).

Influence of antiviral therapy on CD11b/MAC-1, CD16, CD18, and CD62L expression

Treatment with Peg-IFN- α and ribavirin resulted in an increase in the expression of CD11b/MAC-1 and CD18, and a decrease in CD16 and CD62L; however, only the change in

CD62L expression was statistically significant (Fig. 1. and Table 3).

Influence of antiviral therapy on ROS production

After 12 wk of treatment intracellular ROS production of unstimulated neutrophils remained unchanged, but after stimulation with PMA, ROS levels increased statistically significantly. An increase in ROS production was also triggered by the suspension of opsonized *Escherichia coli*; however, these changes were not of statistical significance (Fig. 2 and Table 3).

Influence of antiviral therapy on granulocyte death

Antiviral therapy in patients with HIV/HCV coinfection significantly increased the percentage of apoptotic whole blood neutrophils, whereas the percentage of necrotic cells was not affected (Fig. 3 and Table 3).

Discussion

Impairment of neutrophil function has been demonstrated in both symptomatic and asymptomatic patients with HIV infection (20,21). Neutrophils isolated from HIV-infected patients displayed an impairment of chemotaxis and phagocytosis and dysregulated production of ROS (5,29). Schwartz *et al.* demonstrated that neutrophils extracted from asymptomatic HIV-infected patients had an exaggerated response to LPS and a diminished inhibitory response to S100A8/A9. The authors concluded that this dysregulated response would contribute to the onset of bacterial or fungal infections and increased transactivation of HIV (25). Elbim *et al.* showed that unstimulated PMNs and monocytes from HIV-infected patients are activated even in the early asymptomatic stage of the disease. Unstimulated phagocytes from these patients expressed higher H₂O₂ production and CD11b adhesion molecule expression, and decreased L-selectin expression (8).

In our study we compared PMN oxygen metabolism, the expression of adhesion molecules CD11b/MAC-1, CD16,

TABLE 2. STUDIED PARAMETERS BEFORE PEG-IFN- α AND RIBAVIRIN TREATMENT

			HCV patients (n = 34)	HCV/HIV patients (n = 18)
Adhesion molecules	CD16	%	93.1 (91.0–95.7)	95.1 (91.7–97.1)
		MFI	22728 (20,014–26,912)	20771 (19,462–25,598)
	CD18	%	99.5 (98.9–99.7)	99.7 (99.4–99.8)
		MFI	2589 (2342–3112)	2992 (2354–3683)
	CD62L	%	99.1 (98.5–99.4)	99.0 (96.9–99.3)
		MFI	5956 (5032–8058)	6046 (4541–7933)
ROS production	CD11b/MAC-1	%	99.6 (99.3–99.8)	99.7 (99.4–99.8)
		MFI	6839 (5275–9159)	8299 (7125–11,123)
	Unstimulated	%	0.4 (0.2–0.7)	0.3 (0.2–0.8)
		MFI	1205 (1073–1456)	1386 (1102–1566)
	PMA	%	97.9 (97.0–99.4)	97.8 (93.8–99.4)
		MFI	4148 (3159–5958)	5249 (3722–5875)
Apoptosis	<i>Escherichia coli</i>	%	94.9 (89.2–99.1)	95.8 (92.7–97.3)
		MFI	2973 (2315–3563)	3228 (2634–4096)
	Annexin V(+)	%	16.9 (12.7–21.4)	16.3 (12.3–20.0)
		MFI	2973 (2315–3563)	3228 (2634–4096)
Necrosis	PI(+) Annexin V(-)	%	3.2 (1.9–5.7)	3.0 (1.8–5.2)
		MFI	2973 (2315–3563)	3228 (2634–4096)

HCV, hepatitis C virus; HIV, human immunodeficiency virus; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; MFI, mean fluorescence intensity.

FIG. 1. Flow cytometric analysis of expression of specific surface markers on whole blood granulocytes. Human peripheral whole blood neutrophils were identified and gated based on forward and side scatter (FSC/SSC) dot plot (A). Histograms show CD16 (B), CD18 (C), CD11b/MAC-1 (D), and CD62L (E) expression (expressed as mean fluorescence intensity) on neutrophils before (shaded areas) and after (open areas) 12 wk of combined therapy with Peg-IFN-alfa and ribavirin. Gates (dashed line) were set up according to isotype control data.

CD18, and CD62L, and apoptosis/necrosis of neutrophils in two groups of patients: those infected with HCV alone and those with HIV/HCV coinfection. Moreover, we analyzed the effect of Peg-IFN-alfa and ribavirin treatment on these parameters in HIV/HCV coinfecting patients. In our previous study, similarly to the findings of other authors (7), we ob-

served increased ROS production in patients with HCV monoinfection compared to healthy individuals, whereas in our present study we did not find any differences in ROS production in HCV monoinfected and HIV/HCV coinfecting patients. This may be related to the fact that patients with HIV/HCV coinfection were on cART with undetectable HIV

TABLE 3. STUDIED PARAMETERS IN HIV/HCV COINFECTED PATIENTS BEFORE AND AT WEEK 12 OF TREATMENT WITH PEG-IFN-ALFA AND RIBAVIRIN

			Before treatment (n = 15)	At 12 weeks of treatment (n = 15)
Adhesion molecules	CD16	%	95.1 (91.9–97.1)	94.8 (87.7–96.2)
		MFI	21992 (18,537–26,780)	17407 (16,517–22,566)
	CD18	%	99.7 (99.4–99.8)	99.2 (98.6–99.4)
		MFI	3175 (2700–3791)	3485 (3278–3779)
	CD62L	%	99.0 (96.6–99.3)	97.4 (94.4–97.7)
		MFI	5744 (4392–7933)	*3478 (2915–3949)
ROS production	CD11b/MAC-1	%	99.6 (99.3–99.7)	99.2 (98.1–99.7)
		MFI	9518 (7737–12,632)	11,356 (10,419–13,735)
	Unstimulated	%	0.6 (0.3–1.1)	0.8 (0.3–1.4)
		MFI	1386 (1115–1547)	1380 (1255–1514)
	PMA	%	98.3 (96.7–99.4)	99.4 (98.3–99.8)
		MFI	5346 (4516–6768)	**9990 (8438–12,812)
Apoptosis Necrosis	<i>Escherichia coli</i>	%	95.0 (92.7–96.8)	97.4 (92.4–98.5)
		MFI	3020 (2388–3800)	3448 (2412–4811)
	AnnexinV(+)	%	20.2 (12.4–27.2)	*27.2 (18.2–34.6)
	PI(+) AnnexinV(–)	%	2.5 (1.4–5.0)	2.0 (0.8–3.0)

* $p < 0.05$; ** $p < 0.01$.

PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; MFI, mean fluorescence intensity; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

FIG. 2. Flow cytometric analysis of ROS production by whole blood granulocytes. Human peripheral whole blood neutrophils were identified and gated based on DNA staining (A) and forward and side scatter (FSC/SSC) dot plot (B). Histograms show mean fluorescence intensity of dihydrorhodamine 123 (DHR) staining of unstimulated cells (C), or cells stimulated either with phorbol 12-myristate 13-acetate (PMA; D), or suspension of opsonized *Escherichia coli* (E), before (shaded areas) and after (open areas) 12 wk of combined therapy with Peg-IFN-alfa and ribavirin. Gates (dashed line) were set up according to unstimulated cells.

FIG. 3. Dot plots show percentages of apoptotic/necrotic peripheral whole blood neutrophils gated by forward and side scatter (FSC/SSC) dot plots before and after 12 wk of combined therapy with Peg-IFN-alfa and ribavirin. Gated neutrophils were subdivided into subpopulations based on Annexin V-FITC and propidium iodide (PI) staining characteristics. Subpopulations are defined as Annexin V(−)PI(+) (Q1; necrosis), Annexin V(+)PI(+) (Q2; late apoptosis), Annexin V(−)PI(−) (Q3; viable cells), and Annexin V(+)PI(−) (Q4; early apoptosis).

viral loads, and CD4 T-cell counts were higher than 350 cells/ μ L.

During treatment with Peg-IFN-alfa and ribavirin we observed an increase in ROS production in HIV/HCV coinfecting patients. The same effect of IFN treatment was observed by us and by other authors in HCV-monoinfected patients (6,11,14,15,17). Our present study confirms the results of our previous research conducted in HCV-monoinfected patients (15), which demonstrated that treatment with Peg-IFN-alfa and ribavirin causes activation of PMNs.

Co-occurrence of an increase in ROS production and a decrease in HCV viremia seen after 12 wk of treatment with Peg-IFN-alfa and ribavirin may indicate that neutrophils play an important role in the elimination of HCV infection in HIV/HCV coinfecting patients. Yet the increased ROS production seen during treatment with Peg-IFN-alfa and ribavirin may result in transient transactivation of HIV. Our observations are in accord with the results of other authors, who showed that ROS activate NF- κ B transactivation factor and induce HIV-LTR transactivation in monocytic cell lines (13), and potentiate monocyte production of proinflammatory cytokines (3,12). However, in patients on cART during treatment with Peg-IFN-alfa and ribavirin, we did not observe a transient increase of HIV viremia, which we believe indicates that cART is a safe therapy for use with Peg-IFN-alfa and ribavirin in HIV/HCV coinfecting patients.

Here we conclude that in HIV/HCV coinfecting patients on cART, Peg-IFN-alfa and ribavirin treatment causes activation of neutrophil functions, yet it does not affect the suppression of HIV replication.

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Author Disclosure Statement

No competing financial interests exist.

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